Diphenyleneiodonium Chloride Blocks Inflammatory Cytokine-Induced Up-Regulation of Group IIA Phospholipase A₂ in Rat Mesangial Cells

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ABSTRACT

Inflammatory cytokines, interleukin 1β and tumor necrosis factor-α, potently stimulate rat mesangial cells to express and secrete group IIA phospholipase A₂ (PLA₂). Cytokine-induced up-regulation of PLA₂ has been blocked by inhibitors (antioxidants) of the transcription factor, nuclear factor-κB (NF-κB), suggesting a role for NF-κB in the regulation of group IIA PLA₂ expression. Reactive oxygen species such as H₂O₂, which are elevated in mesangial cells after cytokine activation, can mimic cytokine-induced NF-κB activation. However, the source of reactive oxygen species generation in mesangial cells, produced by cytokine stimulation, has yet to be clarified. Recently, tumor necrosis factor-α has been demonstrated to increase NF-κB expression. To test this hypothesis, we isolated rat mesangial cells and characterized them by ultrastructural and immunochemical methods. This homogeneous mesangial cell population was responsive to cytokine as evidenced by an increase in steady-state levels of group IIA PLA₂ mRNA and extracellular enzymatic activity over time. DPI (0.02–20 μM), added 90 min before cytokine activation, inhibited both group IIA PLA₂ mRNA and enzymatic activity in a concentration-dependent manner. By electrophoretic mobility shift analysis, cytokine activation also increased specific NF-κB binding to one of two NF-κB consensus elements in the rat group IIA PLA₂ promoter and also was suppressed by DPI pretreatment. Antibodies to NF-κB p65 (Rel A) and p50 (but not normal rabbit IgG) supershifted this retardation signal and verified the type of NF-κB species as the classical p50/p65 heterodimer.

Phospholipases A₂ (E.C. 3.1.1.4) are lipolytic enzymes that catalyze bond cleavage at the sn-2 position of glycerophospholipids that produce cis-unsaturated fatty-acids and the corresponding lysodervative (Dennis, 1997). Both enzymatic products are membrane perturbing due to their amphipathic characteristics and can be precursors for potent inflammatory mediators such as eicosanoids and platelet-activating factor (Dennis, 1997). Phospholipase A₂ (PLA₂) enzymes are either calcium dependent or independent with respect to enzymatic activity and are expressed both as cell-associated and secretory forms (Kramer, 1993; Dennis, 1994; Tischfield, 1997). In mammals, five calcium-dependent PLA₂ isozymes have been identified: the cytosolic group IV PLA₂ (Kramer and Sharp, 1997) and four closely related secretory forms group I (Seilhamer et al., 1986), group IIA (Seilhamer et al., 1989), group IIC (Tischfield, 1997), and group V PLA₂ (Tischfield, 1997). Group III is found in bee venom, whereas group IIB is found only in snake venom (Tischfield, 1997). The 14-kDa group IIA PLA₂ is up-regulated by inflammatory cytokines such as interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α) (cytokine) in numerous cells, including astrocytes (Oka and Arita, 1991), vascular smooth muscle cells (Nakama et al., 1990), chondrocytes (Kerr et al., 1989), hepatoma cells (Crowl et al., 1991), and mesangial cells (Nakazato et al., 1991). Only enzymatically active group IIA PLA₂ is proinflammatory and elicits edema when injected into animal paws (Vadas et al., 1989). In addition, group IIA PLA₂ is mitogenic as demonstrated by the proliferation of synovial cells when injected into joints of experimental animals (Va-

ABBREVIATIONS: PLA₂, phospholipase A₂; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; ROS, reactive oxygen species; AA, arachidonic acid; DPI, diphenyleneiodonium chloride; FBS, fetal bovine serum; GM, growth media; DMSO, dimethyl sulfoxide; RT, room temperature; LSM, low-serum media; ACM, activation media; LDH, lactate dehydrogenase.
Group IIA PLA2 (subsequently referred to as PLA2) is thought to play a pivotal role in the pathogenesis of inflammatory kidney diseases such as glomerulonephritis (Wada et al., 1997) due to the enzyme's proinflammatory (Vadas et al., 1989) and mitogenic properties (Vadas et al., 1989; Arita et al., 1991). Indeed, a hallmark of glomerulonephritis is the proliferation of mesangial cells, a resident kidney cell, within the inflammed glomerulus (Sedor, 1992). Cytokines, which may trigger the onset of glomerulonephritis (Lan et al., 1995), up-regulate the synthesis and secretion of PLA2 by cultured mesangial cells (Nakazato et al., 1991). Therefore, delineating the intracellular signaling mechanism(s) for cytokine regulation of PLA2 may lead to future therapeutic drugs aimed at ameliorating the toxic consequences of glomerulonephritis.

Nuclear factor-κB (NF-κB), a cytoplasmically sequestered transcription factor, is activated by cytokines that up-regulate the expression of many inflammatory genes such as IL-β, TNF-α, and IL-6 (Baueerle and Baltimore, 1996). Cytokine activation of cells, including mesangial cells, increases intracellular reactive oxygen species (ROS) such as O2·−, H2O2, and OH− (Radeke et al., 1990; Feng et al., 1995). Exogenously added H2O2 activates NF-κB in cultured cells, indicating ROS as second messengers for inflammatory cytokine signal transduction (Radeke et al., 1990; Feng et al., 1995). Antioxidants and/or heavy metal chelators have successfully blocked inflammatory cytokine-induced NF-κB activation and PLA2 up-regulation (Walker et al., 1995). However, a direct link demonstrating NF-κB as a necessary trans-acting factor regulating transcriptional control for PLA2 has yet to be demonstrated. Therefore, we investigated whether NF-κB binds to the rat PLA2 promoter.

Cytokines have been demonstrated to increase ROS from mitochondria by an acidic-sphingomyelinase-dependent pathway (Schutze et al., 1992). However, embryonic fibroblast cells lacking acidic sphingomyelinase (asmasel−/−) nevertheless demonstrated activated NF-κB upon cytokine treatment (Zumbansen and Stoffel, 1997). This finding suggests that cellular sources other than mitochondrial enzymes may be responsible for cytokine-induced ROS generation and subsequent NF-κB activation. Numerous other cellular sources could potentially contribute to cytokine-induced ROS generation instead of mitochondrial enzymes, such as P-450 complex, nitric oxide synthetase, xanthine oxidase, arachidonic acid (AA) metabolism, and NADPH oxidase (Johnson and Nashr-Esfahan, 1993). Indomethacin (prostaglandin inhibitor) did not block cytokine-induced activation of NF-κB and PLA2 expression in mesangial cells, implying that prostaglandin metabolism is not a major contributor to ROS generation (Vervoordeldonk et al., 1996). Conversely, TNF-α treatment of mesangial cells has been demonstrated to generate superoxide radical (O2·−) production (Radeke et al., 1990). Therefore, we investigated whether a selective NADPH oxidase inhibitor (O'Donnell et al., 1993), dipheneylenedioimid chloride (DPI), could block cytokine up-regulation of PLA2 by inhibiting NF-κB activation.

In this study, a primary mesangial cell line provided evidence that DPI may block cytokine-induced up-regulation of PLA2 in a concentration-dependent manner by suppressing p50/p65 NF-κB binding to one of two NF-κB elements in the rat PLA2 promoter.

### Experimental Procedures

#### Materials

Human recombinant IL-1β and TNF-α were purchased from R&D Systems (Minneapolis, MN). Fetal bovine serum (FBS), glutamine, penicillin, streptomycin, Trizol, and random priming kit were purchased from Life Technologies (Grand Island, NY). Human mesangial cells and media for their growth were purchased by Clonetics (Walkersville, MD). All tissue culture flasks, dishes, and insulin/transferrin/selenium were purchased from Becton Dickinson (Franklin Lakes, NJ). Antibodies for cytokerin, von Willebrand factor, smooth muscle actin, and Thy 1.1 were purchased from Accurate Scientific (Westbury, NY). DPI was purchased from ICN Pharmaceuticals (Costa Mesa, CA). Standard saline citrate buffer (10×) for Northern analysis was purchased from Promega (Madison, WI). Nitrocellulose was purchased from Bio-Rad (Hercules, CA). Radio-nucleotides were purchased from Amersham Corp. (Piscataway, NJ). Oligomers were synthesized by Genosys (The Woodlands, TX). Stainless steel meshes and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

#### Mesangial Cell Isolation

Kidneys from sacrificed male Lewis Sprague-Dawley rats (150 g) were removed, placed in Hanks' solution, and the capsule was removed. All steps were performed aseptically at 4°C unless otherwise stated. The kidney was bivalved and the cortex was separated from the medula. Also, cortical tissue was minced with a glass pestle in Hanks' solution and passed through a 280-μm stainless steel mesh. The suspension of cortex tissue was passed 15 times through a 21-gauge needle and sequentially passed through stainless steel meshes of 280, 190, 140, 90, and 73 μm, respectively. Isolated glomeruli that did not pass through the 73-μm mesh were washed three times with Hank's solution and collected by inverting the mesh and washing with Hanks' solution. By light microscopy, isolated glomeruli appeared spherical, and little-to-no tubular or arteriole tissue was present. Glomeruli were centrifuged for 5 min at 750 g and the pelleted glomeruli saved. Two thousand glomeruli per milliliter were digested with 0.1% collagenase for 30 min at 37°C to remove epithelial cells and centrifuged at 50g for 5 min. The pellet, containing intact glomeruli, was resuspended in growth media (GM) consisting of 82% RPMI 1640, 17% fetal calf serum, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 2 mM glutamine, 60 μg/ml penicillin, and 60 μg/ml streptomycin. Cells were plated in a volume of media that covered only one-third the area of a 100-mm tissue culture dish and incubated at 37°C with 5% CO2. After 5 h, attached glomeruli (10–20%) were washed with PBS, and 6 ml of fresh GM was added.

#### Mesangial Cell Selection and Culture

Isolated rat mesangial cells were grown in GM that was changed every 48 h for the first three passages and every 72 h for passages 4 to 15. To pass cells, 0.025 to 0.5% trypsin in Hanks' solution or PBS was added to detach cells, centrifuged at 300g, and split 1:4. A passage refers to approximately four to five doublings. To select for mesangial cells, isolated cells were 1) grown in d-valine, which inhibits fibroblast proliferation until passage 6; 2) treated for 24 h with 10 μg/ml puromycin, which is toxic to epithelial and endothelial cells; and 3) incubated twice with 0.5% FBS in RPMI 1640 media for 48 h (twice), which detaches epithelial and endothelial cells. Surviving cells in passages 9 to 15 were used for these experiments. Cells (3–5 × 10⁶) were frozen in 50% GM, 40% FBS, and 10% dimethyl sulfoxide, and stored in liquid nitrogen. Cells used in these experiments were frozen up to three times with no noticeable change in their ability to respond to cytokine.
Electron Microscopy

Confuent rat mesangial cells grown in 35-mm dishes were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and stored at 4°C until processed. The fixed cells were washed twice for 5 min at 4°C with 0.1 M sodium cacodylate buffer, pH 7.4. Cells were postfixed for 1 h at 4°C with 2% osmium tetroxide in 0.1 M cacodylate buffer, pH 7, followed by one wash (as above). Cells were then dehydrated with incubations of 50, 70, 80, and 95% ethanol for 10 min at 4°C and then with 100% ethanol for 20 min at room temperature (RT), repeating twice.

Transmission Electron Microscopy

Cells were immersed three times in 100% propylene oxide for 20 min at RT, 50% propylene oxide in 50% poly/bed 812 embedding media (v/v) overnight with constant mixing, followed by pure poly/bed 812 embedding media (v/v) overnight with constant mixing. These prepared cells were embedded into capsules and heated at 60°C for 3 days.

Scanning Electron Microscopy

Cells were immersed three times with hexamethyldisilazane for 20 min at RT, and air dried. The cells were mounted on scanning electron microscope stubs with silver paint, coated with gold-palladium, and stored in a desiccator.

Immunohistochemistry

Para-formaldehyde-perfused rat kidney cortex cross sections were used as positive staining controls for cytokeratin, von Willebrand factor, smooth muscle actin, and Thy 1.1, whereas fixed human mesangial cells were used for positive vimentin staining (data not shown). Isolated rat mesangial cells grown on glass plates were fixed with 4% para-formaldehyde in 0.1 M phosphate buffer, pH 7.2, and stained by the following procedure. Fixed cells were washed three times with PBS for 5 min, blocked with 1.5% serum (horse for mouse monoclonal antibodies, goat for rabbit polyclonal antibodies, 5% BSA for von Willebrand factor), 0.3% Triton X-100 for 1 h, and washed as described above. Primary monoclonal and polyclonal antibodies were used at a concentration between 1 and 5 μg/ml in PBS, 0.5% serum (1.7% BSA for von Willebrand factor), and incubated for 1 h at RT. Cells were washed (as described above) and the appropriate biotinylated secondary antibody applied for 1 h. Hydrogen peroxide quenching was performed with 0.3% H₂O₂ for 30 min. A biotinylated avidin complex was incubated next for 1 h, washed as described above, and washed again with 0.1 M Tris, pH 7.6, three times. One-half milligram per milliliter 3,3-diaminobenzidine in 0.05% H₂O₂ Tris, pH 7.6, was incubated with cells for 2 to 10 min and immediately dehydrated, rehydrated, counterstained with hematoxylin, dehydrated, and mounted. Peroxidase staining was visualized by a reversed-phase Nikon microscope.

Mesangial Cell treatment with IL-1β and TNF-α

Confuent mesangial cells between passages 9 and 15 were grown to confluence with GM in 24-well plates, T-25 or T-75 flasks. GM was aspirated and cells washed twice with PBS, pH 7.4. Low-serum media (LSM), consisting of MCDB media, 0.5% FBS, and 12.5 mM HEPES, was added to cells for 48 h. LSM was aspirated and cells were washed twice with PBS. Activation medium (ACM), consisting of LSM but with 0.1% BSA (fraction V) substituted for FBS, was added with 100 μM IL-1β and TNF-α or PBS and 0.1% BSA, for the indicated time intervals. For experiments with DPI, cells were incubated before the addition of cytokine in ACM with freshly prepared DPI (0.3% DMSO) or 0.3% DMSO for 90 min, aspirated, washed twice with PBS, and then fresh ACM was added. After cytokine incubation, media was collected, centrifuged at 2000g for 5 min at 4°C, and stored at −20°C until assayed. For total RNA isolation, cells were washed twice with PBS, released with 0.5% trypsin and lysed with TRIzol per manufacturer’s instructions.

Measurement of PLA₂ Activity

PLA₂ activity was measured by an established method that used autoclaved 1-[14C] oleate-labeled Escherichia coli (E. coli; Dorsam et al., 1995). The reaction mixtures contained: 10 nmol of labeled E. coli phospholipid (4000–6000 cpm), 1 mM CaCl₂, 100 mM Tris, pH 7.4, and 100 μl of conditioned mesangial cell media. Media was diluted appropriately with ACM to ensure linear kinetics. Samples were extracted by the following procedure. Reactions were stopped with 3 ml of chloroform/methanol (1:2 v/v) and vortexed briefly. One milliliter of chloroform and 4 ml of H₂O were added and vortexed for 15 s. The samples were centrifuged at 500g for 5 min at RT. The organic layer was collected and dried under nitrogen gas at 37°C. The remaining lipid residue was dissolved in 65 μl of chloroform/methanol (1:9 v/v) and separated on silica plates with a running buffer of 78.5% petroleum ether, 19% ethyl ether, and 2.5% glacial acetic acid.

The regions corresponding to fatty acid and phospholipid were scraped into scintillation vials containing nonaqueous scintillation cocktail, and the counts per minute of 14C was measured. Hydrolysis was calculated as the counts per minute of oleate divided by the sum of counts per minute of oleate and E. coli phospholipid. This ratio was multiplied by the dilution factor, 10 nmol of phospholipid, time (minutes), and volume assayed (milliliters) to obtain an enzymatic rate with units of nanomoles of phospholipid hydrolyzed per minute per milliliter.

Northern Analysis

Confuent mesangial cells in T-75 flasks were treated with cytokine for the indicated time interval, which were lysed by TRIzol, and total RNA isolated per manufacturer’s instructions. RNA concentration was measured by spectrophotometry (260 nm) and 10 to 25 μg of total RNA was loaded per lane on a 1.25% agarose, 2.2 M formaldehyde gel, and electrophoresed overnight with 15 V (constant voltage) for 16 h at RT. The RNA gel was removed, blotted (16 h) by capillary action with 10× standard saline citrate buffer onto nitrocellulose and baked for 2 h at 80°C. The distance of ribosomal RNA migration was measured. Group IIA PLA₂ transcript is ~800 base pairs. Twenty nanograms of group IIA PLA₂ cDNA and β-actin (control gene) were labeled with alpha [32P]dCTP by the random priming procedure. Percentage of incorporation of radiolabeled nucleotide was between 15 and 50%, and the entire synthesized probe was used for hybridization studies.

The nitrocellulose membrane was blocked with salmon sperm DNA in 40 mM phosphate buffer, 1% SDS, and 5 mM EDTA for 1 h at 65°C in a hybridization oven (25a). The buffer was decanted and fresh buffer with alpha [32P]dCTP-labeled probe was added to the membrane and incubated for 16 h. Membranes were exposed to X-ray film with an intensifying screen at ~80°C until developed (24 h) or by phosphoimager.

Electrophoretic Mobility Shift Assay

Annealing of Oligomers. Synthesized sense and antisense DNA oligomers were annealed by placing equal molar amounts in a 1.5-ml microfuge tube, heated to 90°C, and allowed to cool slowly. The annealed oligo was electrophoresed on a 20% acrylamide gel with 150 V. Probe was visualized by UV-shadowing and eluted in 10 mM Tris and 1 mM EDTA (TE buffer) overnight at 4°C, and absorbance values (260 nm) were measured to determine oligonucleotide concentration. Probe was stored at −20°C until needed.

Isolation of Nuclear Extracts. Confuent rat mesangial cells in T-75 flasks were treated with cytokine for the indicated time intervals. Cells were washed, scraped with ice-cold PBS, and collected by centrifugation (300g). All subsequent steps were conducted at 4°C unless otherwise noted. Cells were lysed with 100 μl of lysis buffer containing 20 mM Tris, pH 7.4, 140 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.5% NP-40, 0.5 mM sodium orthovandate, 1 mM aprotinin, 1 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride, and centrifuged at 12,000g for 5 min.
The pelleted nuclei were washed twice with 1 ml of lysis buffer lacking NP-40 and resuspended in 50 μl of nuclear extraction buffer containing 250 mM Tris, pH 7.8, 60 mM KCl, 1 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM sodium orthovanadate. After freezing and thawing three times, the nuclei were centrifuged at 12,000 g for 15 min and the supernatants (nuclear extracts) were frozen until assayed. Protein concentrations were determined by the Bradford method.

DNA Binding Assay. NF-κB consensus sequences (90% homologous NF-κB sequence, 5′-GGAAAGGGGAAATTACCAAGG-3′; 80% homologous NF-κB sequence, 5′-GCGTGGGTATGGCCCGTCG-3′; Promega NF-κB consensus sequence, 5′-GGAAGGACTTTCGCTCTG-3′) were labeled with T4 polynucleotide kinase with [γ-32P]ATP and incubated for 30 min at RT. Unincorporated nucleotide was removed by phenol/chloroform extraction and ethanol precipitation. The binding reaction consisted of 5 μg of protein, 5% glycerol, 1 μg of poly(dI-dC), and 0.1 ng 32P-labeled NF-κB oligonucleotide (binding reaction), which was incubated for 30 min at RT. The protein-DNA complexes were then resolved on a 5% polyacrylamide gel electrophoresis gel, 0.5 × Tris-borate-EDTA buffer with 40 V. Gels were dried in a glycerol/ethanol mixture and exposed to X-ray film or a phosphoimager screen.

Competitive studies were conducted by adding increasing concentrations of unlabeled probe to the binding reaction. The effect of DPI on NF-κB binding was carried out by exogenously adding DPI to the binding reaction (minus labeled probe) for 10 min at RT before adding the labeled probe. Supershift analysis used 1 μg of anti-p65, -p50, or normal rabbit IgG added to the binding reaction for 30 min at RT.

Viability Analysis

Lactic Dehydrogenase Activity Assay. Lactate dehydrogenase (LDH) activity in rat mesangial cellular extracts and mesangial cells conditioned media was measured. Briefly, 0.1 M NADH, 0.1 mg/ml sodium pyruvate, PBS, pH 7.4, was incubated with mesangial cellular extracts or mesangial cells conditioned media (5–10% total volume) for 20 min at RT, and the decrease in absorbance at 340 nM was monitored with a spectrophotometer. All measurements ensured linear kinetics because two enzyme concentrations were analyzed per sample. Twenty micromolar DPI pretreatment (90 min) did not significantly modulate LDH activity levels compared with control and were always <10% total LDH activity (standardized for cell number).

Trypan Blue Exclusion. On ice, cells were resuspended in RPMI, diluted (1:2) with 0.2% trypan blue, and counted on a hemocytometer. Up to a 20 μM DPI pretreatment (90 min) did not significantly modulate the extent of trypan blue exclusion compared with control after 24 h, and all experiments consisted of ≥85% viable cells.

Statistical Analysis

SAS institute (version 6) statistical procedures were performed by the Department of Biostatistics at Virginia Commonwealth University. Percentage of inhibition by DPI versus control was analyzed for statistical significance by one-way parametric analysis. A P value of <.05 was considered statistically significant.

Results

Mesangial Cell Characterization. Isolated glomerular cells used for these experiments required extensive characterization to properly identify a mesangial cell phenotype and to determine the homogeneity of the cell culture. Surviving cells in passages 9 to 15 showing an appropriate stellate mesangial cell morphology (Radeke et al., 1990) were characterized ultrastructurally and immunochemically. Consistent with a mesangial phenotype, scanning and transmission electron micrographs in Fig. 1 confirmed a large nucleus to cytoplasm ratio, numerous nucleoli, a distinct rough endoplasmic reticulum, and Golgi apparatus (Lan et al., 1995). Mesangial cells are mesenchymal in origin and have smooth muscle characteristics and therefore express vimentin and smooth muscle actin (Radeke et al., 1990). In addition, it has recently been reported that rat mesangial cells are positive for the plasma membrane protein Thy 1.1 (Radeke et al., 1990). Table 1 shows the high percentage of cells stained positive for vimentin, smooth muscle actin, and Thy 1.1.

Fig. 1. Electron microscopy of isolated mesangial cells. A, scanning electron micrograph of a confluent mesangial cell monolayer. Arrows indicate electron micrograph of a confluent mesangial cell monolayer. Arrows indicate large nucleus (circle) with numerous nucleoli (smaller circles). B, transmission electron micrograph of two mesangial cells. Arrows indicate the presence of a distinct Golgi apparatus and endoplasmic reticulum. All of these characteristics are representative of a mesangial cell phenotype.
Biochemical Characterization of Mesangial Cells. Biochemical characterization of cultured mesangial cells was performed by treatment with 100 pM IL-1β and TNF-α for 50 h. Figure 2A illustrates the enzymatic increase in PLA2 activity from conditioned medium as measured by 1-[^14]C oleate-labeled E. coli. Secreted PLA2 activity was not detected from cells treated with vehicle alone. PLA2 mRNA levels showed a time-dependent increase similar to PLA2 enzymatic activity as measured by Northern analysis (Fig. 2B), indicating that the increase in secreted enzymatic activity is associated with an elevation in steady-state levels of PLA2 mRNA. Figure 2C illustrates the concentration dependence of cytokine on secreted PLA2 enzymatic activity. IL-1β or TNF-α alone increased the expression of PLA2, whereas both IL-1β and TNF-α up-regulated enzyme expression and subsequent secretion to a greater extent than either one alone. Synergistic up-regulation by both cytokines for PLA2 (mRNA, protein mass and enzymatic activity) has been previously demonstrated (Konieczkowski and Sedor, 1993). IL-2 and IL-4 did not activate rat mesangial cells based on secreted PLA2 enzymatic levels in conditioned media (data not shown).

Diphenyleneiodonium Chloride Inhibits Cytokine-Induced PLA2 Expression. Vervoordeldonk et al. (1997) have reported that cytokine-induced up-regulation of PLA2 in rat mesangial cells is blocked by the antioxidant pyrrolidine dithiocarbamate, a known NF-κB inhibitor. Pyrrolidine dithiocarbamate lowered nuclear NF-κB binding to its consensus sequence by presumably neutralizing the elevation of ROS generation in response to cytokine and inhibiting NF-κB activation (Baueuler and Baltimore, 1996). However, the cellular source for cytokine-induced ROS generation in mesangial cells is not clearly defined. Recently, NADPH oxidase has been implicated as a potential source for cytokine-induced generation of ROS (Radeke et al., 1990). Therefore, DPI, a selective inhibitor of NADPH oxidase (O’Donnell et al., 1993), was used and its effect on cytokine-induced up-regulation of PLA2 was investigated. Figure 3A illustrates the effects of a 90-min exposure to DPI on the up-regulation of PLA2 in a range of 0.02 to 20 μM. The decrease in PLA2 enzymatic activity agreed well with a concomitant reduction in mRNA transcript as seen in lanes 3 and 4 of Fig. 3B. These data demonstrate that DPI pretreatment suppresses steady-state levels of PLA2 mRNA, resulting in a decrease in subsequent enzyme synthesis and secretion.

Mechanism of Inhibition for DPI on PLA2 Up-Regulation. To determine whether NF-κB was a viable candidate for directly enhancing the transcriptional rate of PLA2, we analyzed 500 base pairs of the rat PLA2 promoter region for known transcription factor-binding motifs (Seilhamer et al., 1989). This analysis revealed two potential NF-κB-binding sites located at 112 and 131 base pairs upstream from the transcriptional start site (Fig. 4A). The −112 motif is 80% homologous, and the −131 motif is 90% homologous to the known NF-κB (p50/p65) consensus sequence. Interestingly, as seen in Fig. 4C, electrophoretic mobility shift analysis demonstrated no detectable retardation signal with the −112
sequence from cells activated with cytokine. However, the −131 sequence as well as a commercially available NF-κB consensus probe (Fig. 4, B and D; Promega) resulted in the detection of a retardation signal that peaked at 40 min and persisted for 24 h. Cells pretreated with DPI (0.2 and 20 μM) and then activated with cytokine for 1 and 24 h showed a decrease in this retardation signal when the −2131 probe and the NF-κB probe were used (Fig. 4, B and D; lanes 5 and 6 compared with 4, and lanes 8 and 9 compared with 7). In addition, the decrease in NF-κB binding observed at 60 min, due to DPI pretreatment, may account for the decrease in steady-state levels of PLA2 mRNA and enzymatic activity as seen in Fig. 3, A and B. These data suggest that DPI blocks protein binding to an NF-κB element found in the rat PLA2 promoter and may explain the observed inhibition by DPI on cytokine-induced up-regulation of this mitogenic and proinflammatory enzyme.

Identification of Protein Species Binding to −131 NF-κB Sequence. Competition and supershift studies were conducted to identify the protein(s) responsible for the observed retardation signal. Competition and supershift studies were conducted to identify the protein(s) responsible for the observed retardation signal. Nuclear extracts were isolated as described in Experimental Procedures and incubated with γ[32P]ATP-labeled −131 sequence (B), −112 sequence (C), and NF-κB consensus sequence (D).

Discussion

The response of mesangial cells to individual cytokines is well documented. Typically, these studies use nanomolar concentrations. In contrast, both IL-1β and TNF-α were used in these experiments instead of a single cytokine treatment.
for two reasons. First, both cytokines are direct mediators of glomerulonephritis and are present simultaneously during the progression of this disorder (Sedor, 1992; Lan et al., 1995). Therefore, the addition of IL-1β and TNF-α at subnanomolar concentrations is not only physiologically relevant but also mimics more closely the environment of the inflamed glomerulus (Sedor, 1992). Second, although there are many similarities between IL-1β and TNF-α-induced signal transduction, there are definite differences pertaining to elicited cellular responses (Feng et al., 1995). Thus, inhibiting a cellular response induced by IL-1β may not inhibit TNF-α responses, and conversely. However, inhibiting the synergistic effects induced by both cytokines may be more meaningful in regard to glomerulonephritis.

The classical p50/p65 NF-κB element binds to a 90% homologous NF-κB element located at position −131 in the rat PLA2 promoter but not to an 80% homologous NF-κB element located at position −112. To our knowledge, this is the first demonstration of specific NF-κB binding to identical DNA sequences found in the promoter of rat PLA2. The affinity of p50/p65 for the −131 sequence appears less than that for the NF-κB consensus sequence as demonstrated in the competition study because less unlabeled NF-κB consensus sequence was needed to completely ablate the retardation signal compared with unlabeled −131 sequence. This is an expected consequence due to the −131 sequence sharing only 90% homology with the NF-κB consensus sequence. Furthermore, Konieczkowski and Sedor (1993) demonstrated that IL-1β increased the transcriptional rate for PLA2 only modestly (2-fold) but was amplified by a prolonged half-life for its mRNA transcript. An extended half-life of Cox II mRNA message, also up-regulated by IL-1β, also has been observed (Srivastava et al., 1994). Therefore, a single low-affinity NF-κB-binding site directing PLA2 transcription may account for the moderate transcriptional increase observed for PLA2 when activated by cytokine. However, because only 500 nucleotides of the rat PLA2 promoter sequence were analyzed, it is possible that additional NF-κB-binding elements exit further upstream that may contribute to NF-κB-directed transcription.

The biphasic inhibition by increasing concentrations of DPI on p50/p65 binding may indicate that NF-κB is necessary but not sufficient for cytokine-induced up-regulation of PLA2, and thus other mechanisms besides NF-κB may contribute to cytokine-induced PLA2 expression. Because the PLA2 mRNA message is completely ablated at 20 μM, perhaps different transcription factors such as activator protein-1 (AP-1) need to form a suitable transcription complex for RNA polymerase II-initiated transcription. Scheinman et al. (1995) have demonstrated that NF-κB associates with other transcription factors that are necessary for transcriptional activation. A second explanation may be that multiple signaling pathways, simultaneously activated by cytokine, need to converge for NF-κB-directed transcriptional activation. This idea is supported by the observation made by Bergmann et al. (1998) that TNF-α-induced NF-κB translocation is not sufficient for NF-κB-directed transcriptional activity. A third explanation could be that the stability of PLA2 mRNA (Sedor et al., 1993) may be significantly attenuated by higher concentrations of DPI, thus not allowing for a modest increase in transcriptional activity (2-fold) to be measured by Northern analysis. There is no evidence, however, to suggest that DPI can alter mRNA stability.

NF-κB binding was induced by IL-1β and TNF-α within 20 min and persisted through 24 h. Moreover, NF-κB binding peaked at 40 to 60 min, declined between 2 and 6 h, and subsequently peaked for a second time at 18 and 24 h (data not shown) However, another group also has demonstrated NF-κB binding 24-h postcytokine activation of rat mesangial cells (Vervoordeldonk et al., 1997). Consequently, these data may suggest an autocrine activation mechanism initiated by inflammatory cytokines and perpetuated by the up-regulation of IL-1β and TNF-α. A perpetual activation of NF-κB may explain, at the molecular level, the inflammatory loop taking place within mesangial cells during glomerulonephritis. Uncoupling this overzealous inflammatory reaction may unlock the glomerulus from this autocrine loop and allow the healing process to begin.

However, targeting NF-κB in an attempt to regulate chronic inflammation may cause inadvertent complications. Targeting instead, mechanisms other than NF-κB activation, such as mRNA stability of target transcripts, for example, although maintaining an intact NF-κB pathway and high expression of necessary antiapoptotic proteins [TRAF 1, TRAF 2, C-IAP1, C-IAP2, and IEX-1L (Irani et al., 1997)] also may regulate chronic inflammation with fewer deleterious side effects. Recently, it has been demonstrated that TNF-α-induced activation of NF-κB up-regulates the expression of antiapoptotic proteins capable of rescuing cells from programmed cell death (Irani et al., 1997) Hence, the blocking of NF-κB translocation to the nucleus may induce an undesired apoptotic event in cells proximal to an inflammatory foci.
To our knowledge, this is the first demonstration that a DPI compound can block cytokine-induced up-regulation of PLA₂ and lower p50/p65 NF-kB binding. DPI is a selective NADPH oxidase inhibitor (O’Donnell et al., 1993) that irreversibly inhibits enzymatic activity and DPI has been used by others to block NADPH oxidase activity and superoxide generation. However, DPI also inhibits other flavo-proteins capable of catalyzing the generation of ROS such as NADH oxidase, inducible nitric oxide synthase, and mitochondrial NADH dehydrogenase (O’Donnell et al., 1993). Therefore, the requirement for NADPH oxidase activity in IL-1β and TNF-α-induced activation of NF-kB and PLA₂ up-regulation is indirect. The mitochondria has been suggested to be a major source for ROS necessary for NF-kB activation (Schutze et al., 1992). We speculate, however, that plasma membrane flavo-proteins (NADPH oxidase) will be inhibited more readily and to a greater extent compared with flavo-proteins found intracellularly (mitochondrial NADH dehydrogenase) after only 90 min. Furthermore, we surmise that if mitochondrial NADH dehydrogenase were irreversibly inhibited by DPI, then, the cell’s ability to generate ATP would be significantly blunted, causing cellular death. Yet, because a short pretreatment of DPI (90 min) did not decrease the high percentage of viable cells compared with control (<85%), we conclude that DPI is not inhibiting mitochondrial NADH dehydrogenase.

Recently, an eloquent study (Thommesen et al., 1998) showed that inhibitors for cytoplasmic PLA₂ (group IV) and secretory PLA₂ (groups IIA and V) block TNF-α-induced up-regulation of NF-kB and intercellular adhesion molecule expression in epithelial cells. They suggest the involvement of arachidonic acid in the activation of NF-kB. Furthermore, group IV PLA₂ has been shown to be phosphorylated (ser/thr) within minutes by IL-1β activation of mesangial cells (Gronich et al., 1994). Indeed, nonspecific phospholipase inhibitors (arachidonic acid and p-bromoacylbromide) have previously been demonstrated to block cytokine-induced up-regulation of IL-6 (Sedor et al., 1993). Moreover, Sedor et al. (1993) have demonstrated that AA and ROS are elevated on cytokine activation of mesangial cells. A possible mechanism involving AA and ROS could exist contributing to the activation of NF-kB by a mechanism similar to how the neutrophil cell undergoes its respiratory burst phase. It has been shown that blocking cPLA₂ activity in neutrophil cells abolishes NADPH oxidase activity and superoxide generation (Dana et al., 1994). Exogenously added AA and other cis-unsaturated fatty acids, but not saturated fatty acids, restored superoxide generation (Dana et al., 1994). Therefore, we propose that mesangial cells may respond to inflammatory cytokines by activating NF-kB and up-regulating several genes, including PLA₂, through the concerted activities of group IV PLA₂ and NADPH oxidase. In summary, this study demonstrates that IL-1β and TNF-α-induced up-regulation of PLA₂ is blocked by DPI (0.02–20 μM) in a concentration-dependent fashion at both the mRNA and protein levels in primary rat mesangial cells. Cytokine treatment induced p50/p65 NF-kB binding to the −131 sequence but not to the −112 sequence located in the rat PLA₂ promoter, and DPI suppressed the binding of NF-kB p50/p65 heterodimer at the −131 sequence. We conclude from the results of our study that IL-1β and TNF-α-induced up-regulation of PLA₂ is mediated, in part, through the binding of NF-kB p50/p65 to the −131 sequence located in the rat PLA₂ promoter. Furthermore, our study supports that ROS generation induced by cytokine-receptor interaction may be generated by several cellular enzymes such as NADPH oxidase in addition to respiratory mitochondrion enzymes. Therefore, drugs directed at inhibiting NADPH oxidase activity may lead to novel therapeutic strategies for the treatment of inflammatory kidney diseases.

References


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